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Published in:
Journal of Bacteriology

DOI:
10.1128/JB.00473-07

Publication date:
2007

JOURNAL OF BACTERIOLOGY, July 2007, p. 5210–5222 Vol. 189, No. 14
0021-9193/07/$08.00+0 doi:10.1128/JB.00473-07

Identification of a Novel Streptococcal Gene Cassette Mediating SOS Mutagenesis in *Streptococcus uberis*▽

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Received 29 March 2007/Accepted 8 May 2007

Streptococci have been considered to lack the classical SOS response, defined by increased mutation after UV exposure and regulation by LexA. Here we report the identification of a potential self-regulated SOS mutagenesis gene cassette in the *Streptococcaceae* family. Exposure to UV light was found to increase mutations to antibiotic resistance in *Streptococcus uberis* cultures. The mutational spectra revealed mainly G:C→A:T transitions, and Northern analyses demonstrated increased expression of a Y-family DNA polymerase resembling UmuC under DNA-damaging conditions. In the absence of the Y-family polymerase, *S. uberis* cells were sensitive to UV light and to mitomycin C. Furthermore, the UV-induced mutagenesis was almost completely abolished in cells deficient in the Y-family polymerase. The gene encoding the Y-family polymerase was localized in a four-gene operon including two hypothetical genes and a gene encoding a HdiR homolog. Electrophoretic mobility shift assays demonstrated that *S. uberis* HdiR binds specifically to an inverted repeat sequence in the promoter region of the four-gene operon. Database searches revealed conservation of the gene cassette in several *Streptococcus* species, including at least one genome each of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mitis*, *Streptococcus sanguinis*, and *Streptococcus thermophilus* strains. In addition, the *umuC* operon was localized in several mobile DNA elements of *Streptococcus* and *Lactococcus* species. We conclude that the *hdiR-umuC*-ORF3-ORF4 operon represents a novel gene cassette capable of mediating SOS mutagenesis among members of the *Streptococcaceae*.

Organisms from all kingdoms of life avail of complex responses to protect cells from deleterious consequences of DNA damage caused by, e.g., UV radiation as well as some classes of antibiotics (23). The cellular physiological response to insult genetic integrity, i.e., SOS response, was first recognized in *Escherichia coli* more than three decades ago (52). In *E. coli*, the expression of SOS genes is controlled by a repressor protein, LexA, as well as by RecA via its inducible co-protease activity. In response to DNA damage, activated RecA induces autocatalytic cleavage of the Ala^{84}-Gly^{85} bond of LexA, which disrupts its ability to dimerize (35, 57). Self-cleavage of LexA inactivates LexA as a repressor, which induces the expression of SOS genes. SOS mutagenesis is mainly mediated by error-prone polymerases and takes place under severe conditions where nonmutagenic repair processes are insufficient to ensure DNA replication over lesions (23). In the process of translesion synthesis (TLS), a DNA polymerase incorporates nucleotides opposite noninstructional or misinstructional DNA lesions. In *E. coli*, three SOS-induced genes encode DNA polymerases that perform TLS (42). The Y-family polymerases PolIV (DinB), encoded by *dinB*, and PolV ($UmuD_2'C$), encoded by the *umuDC* operon, and the B-family polymerase, encoded by the *polB* gene, improve the ability of cells to survive DNA damage by synthesizing through DNA lesions that block replication forks (63).

In *E. coli*, almost all SOS-targeted UV mutagenesis results from the activity of PolV (53, 64), and the *umuDC* operon is the only SOS locus that must be induced for SOS mutagenesis (61). PolV consists of one molecule of UmuC together with two molecules of activated UmuD (UmuD′) that arise from RecA-mediated self-cleavage of native UmuD in a reaction resembling that of LexA (12). According to phylogenetic analysis, the UmuC subfamily of Y polymerases can be further subdivided into gram-negative and gram-positive branches (45). The *umuC*-like genes of gram-negative bacteria are characteristically regulated by LexA and always expressed in an operon with a *umuD*-like gene that has not been identified in gram-positive bacteria. With the notable exceptions of YqjW and YqjH of *Bacillus subtilis* (19, 62) and SACOL1400 of *Staphylococcus aureus* (16), there is little functional data on UmuC-like proteins from gram-positive bacteria. Thus, it remains to be determined which of the gram-positive orthologs identified in silico (45) are indeed functional homologs of *E. coli* UmuC.

Notably, TLS not only provides the cell with an immediate escape from DNA replication blocks but also is a major mechanism in adaptive mutation. Bacteria appear to be able to adjust their mutation rates depending on environmental conditions and, consequently, also speed up their adaptation to hostile conditions (6, 54). One outcome of genetic variation in bacterial populations is the evolution of resistance to antibiotics. SOS-driven adaptive mutation and the spread of antibiotic resistance through SOS-triggered induction of mobile DNA

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▽ Published ahead of print on 18 May 2007.

elements pose a major threat to public health and motivate studies of SOS-like mechanisms in pathogenic bacteria (28). Several antibiotics, such as ciprofloxacin (50), trimethoprim (32), rifamycins (14), and β-lactams (39), are known to induce an SOS response that increases mutations and accelerates the evolution of antibiotic resistance in *E. coli* (14). Furthermore, it has been shown that the prevention of induction of the LexA-regulated SOS response inhibits the evolution of antibiotic resistance in *E. coli* (14). Thus, inhibitors of SOS-induced polymerases present an appealing strategy to combat the evolution of antibiotic resistance (14). Understanding of the mutagenic responses in diverse pathogens is crucial for such pharmacological approaches. In *Vibrio cholerae*, the ciprofloxacin-induced SOS response increased the expression of genes necessary for transfer of the integrating conjugative element SXT (3). SXT carries several antibiotic resistance genes, and the transfer of this element to other strains of *V. cholerae* promotes the spread of antibiotic resistance. In *S. aureus*, antibiotic-induced SOS responses affect virulence by modulating mobile genetic elements and affecting chromosomal virulence gene expression (4, 25, 37, 67). Remarkably, SOS-induced error-prone DnaE2 polymerase, by its mutagenic activity, contributes to the ability of *Mycobacterium tuberculosis* to persist and develop antibiotic resistance during infection (9).

The genus *Streptococcus* includes species that are part of the normal microbiota of animals and humans, although several members of this genus are important pathogens. *Streptococcus uberis* is an environmental mastitis pathogen and a member of the pyogenic group of streptococci. In a recent study, 51% of *S. uberis* infections failed to respond to conventional antibiotic treatment and most of these infections were found to be persistent (40), suggesting the adaptability of this pathogen. At present, the mechanisms of adaptive mutagenesis in *S. uberis*, as well as in other *Streptococcus* species, are largely unexplored. Streptococcal species lack LexA, and even the existence of a classical SOS response, defined by increased mutation after UV exposure, has remained questionable (24). However, streptococci are equipped with polymerases capable of mutagenic DNA repair. For example, in *Streptococcus pyogenes*, essential DnaE has been characterized as a highly error-prone DNA polymerase capable of TLS in vitro (11). Recently, *Streptococcus pneumoniae* cells were shown to respond, upon exposure to the DNA-damaging agent mitomycin C, by RecA-dependent induction of the *com* regulon and genetic transformability (51). It was demonstrated that in this bacterium competence could play a role similar to that of the SOS response in *E. coli* (51). However, as noted by Cirz and coworkers (15), genomes of *S. pneumoniae* do encode Y-family polymerases, which could contribute to induced mutagenesis and genetic diversification. Previously, Tn*5252*, a streptococcal conjugative transposon carrying genes capable of encoding coding proteins resembling UmuC and UmuD, has been reported to confer a mutagenic response in recipient cells (41). In our previous study, the genome of a nonpathogenic member of the *Streptococcaceae*, *Lactococcus lactis*, was searched for the presence of UmuD-like proteins (58). Functional analysis of the closest homolog of the UmuD-like protein of Tn*5252*, encoded by *ynaB* in *L. lactis*, revealed that YnaB carries an N-terminal helix-turn-helix (HTH) motif for DNA binding (58). Furthermore, it was found that YnaB is in fact a LexA-like regulator that was named HdiR (*h*eat and *D*NA damage *i*nducible *r*egulator) (58). Unlike LexA, the N-terminal cleavage product of HdiR still binds to its target sequence and loses its repressor activity only after ClpP-dependent degradation. Thus, the activity of HdiR is controlled by both RecA and ClpP (22, 58).

In the present study, we investigated *S. uberis* for the presence of mechanisms enabling adaptive mutagenesis and evolution of antibiotic resistance. We found a small DNA damage-inducible cassette including a gene encoding an error-prone DNA polymerase. Inactivation of the gene encoding the DNA polymerase revealed that this gene cassette promotes UV-inducible mutations to antibiotic resistance. The gene cassette was localized in several *Streptococcus* genomes and mobile elements. We propose that the novel mutagenesis gene cassette identified in this study represents a streptococcal SOS response unit.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture growth conditions. The strains and plasmids used in this study are listed in Table 1, and the oligonucleotides used are provided in Table 2. *S. uberis* ATCC BAA-854 (0140J) is a well-characterized bovine mastitis strain (26, 31, 60). The *S. uberis* strains were grown routinely at 37°C using TSYE agar (3% [wt/vol] Trypticase soy broth, 0.3% [wt/vol] yeast extract, 1.5% [wt/vol] Bacto agar) or THY broth (Todd-Hewitt broth with 1% [wt/vol] yeast extract), without shaking, supplemented with 1 μg/ml tetracycline when needed. *E. coli* strains were cultivated at 37°C in Luria-Bertani (LB) medium (55) supplemented with 5 μg/ml tetracycline when needed. The antibiotic concentrations used for the screening of *S. uberis*-resistant mutants were 2 μg/ml of ciprofloxacin (MP Biomedicals LLC, Germany) and 2 μg/ml of rifampin (Sigma).

MIC tests were performed using Etest strips (AB Biodisk, Sweden). *S. uberis* cells grown overnight on a TSYE agar plate and suspended in THY broth were used to seed fresh TSYE agar plates prior to placing Etest strips in the middle of the plate. Plates were incubated overnight, and results were interpreted according to the instructions provided by the manufacturer (AB Biodisk, Sweden). The MIC was defined as the lowest concentration of the antibiotic that inhibited growth.

UV-induced mutagenesis. The appropriate UV dose for *S. uberis* cells was determined as follows. Overnight cultures were diluted 1:500 in THY broth and grown at 37°C until an optical density at 600 nm (OD_{600}) of 0.2. Then, 2-ml aliquots were centrifuged (4,500 × *g*, 10 min, room temperature), and pelleted cells were resuspended in 10 ml of 0.9% NaCl. Cell suspensions were transferred to petri dishes and subjected to UV light dosages of 0, 25, or 50 J/m^2 using a Spectrolinker XL-1000 UV cross-linker (Spectronics Co.). Cells were plated immediately after UV treatment on TSYE agar and incubated overnight at 37°C. Cell viability was determined by counting the number of colonies. Using this protocol for the *S. uberis* ATCC BAA-854, the highest UV dose used (50 J/m^2) was found to result in 20% (standard deviation, ±6.7%) cell survival, calculated from three independent experiments. To determine UV-induced antibiotic resistance, UV-irradiated cells were harvested as described above, resuspended in 15 ml of THY broth, and allowed to grow overnight at 37°C. Overnight cultures were plated onto TSYE agar (cell viability) and agar supplemented with 2 μg/ml rifampin or 2 μg/ml ciprofloxacin. Plates were grown overnight at 37°C, and the numbers of colonies were counted. The proportion of mutations was the ratio of resistant cells/viable cells. Determination of the proportions of rifampin- and ciprofloxacin-resistant cells was conducted from at least three independent cultures.

Pyrosequencing. For pyrosequencing of ciprofloxacin resistance-determining mutations, *S. uberis* cells were grown and UV induction was performed as described above. Following UV induction, the cultures were divided into 0.5-ml aliquots and grown overnight. Each aliquot was plated onto TSYE agar supplemented with 2 μg/ml ciprofloxacin. After incubation for 2 days at 37°C, a single ciprofloxacin-resistant colony from each plate was picked for sequencing analysis to ensure independent mutational events. DNA regions covering bases 228 to 255 and 293 to 314 from the *parC* gene were analyzed by pyrosequencing of ciprofloxacin-resistant wild-type (wt) and EH58 clones to determine the mutation frequency and mutation types present in the selected DNA region.

TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference
Strains		
S. uberis		
ATCC BAA-854 (0140J)	Genome sequencing strain (sequence available in http://www.sanger.ac.uk/Projects/S_uberis/)	ATCC
EH 78	ATCC BAA-854 derivative containing pEH55, Tet^r	This work
EH 58	ATCC BAA-854 derivative Δ*umuC* (1.4-kb deletion) Tet^s	This work
EH 79	EH58 containing pEH79, Tet^r	This work
EH 80	EH58 derivative, Tet^s, with *umuC* complement	This work
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB laqI^{q}ZΔM15*]	Promega
EH55	JM109 containing pEH55	This work
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI^{q}ZΔM15 Tn*10(Tet^r)]^c	Stratagene
Plasmids		
pGhost8	Tet^r, a thermosensitive derivative of pGK12	Maguin et al. (36)
pEH55	Tet^r, pGhost with 1.0-kb XbaI-PstI-SalI fragment containing 1.4-kb in-frame deletion of *umuC*	This work
pEH79	Tet^r, pGhost with 3.0-kb XbaI-EcoRI fragment containing *hdiR-umuC*-ORF3-ORF4 operon without the upstream region	This work
pBluescript-II SK+	Am^r, cloning vector	Stratagene
pQE-30	Am^r, vector for overexpression of proteins with N-terminal His_6 tag	QIAGEN
pQE-*hdiR*	Am^r, pQE derivative for overexpression of His-tagged HdiR	This work
pBluescript-IR	Am^r, *E. coli* vector carrying 26-bp fragment containing the IR preceding the *hdiR*	This work
pBluescript-ctrl	Am^r, *E. coli* vector carrying 26-bp fragment from the region preceding the *hdiR* (not containing the IR)	This work

PCR amplification of part of the *par*C gene was performed with primer pair p1/p2, in which p2 was biotinylated. Pyrosequencing was performed using streptavidin-coated Sepharose beads (Amersham Biosciences), a PSQ 96 MA instrument, a vacuum prep workstation, and Pyro Gold SQA reagents (Biotage AB, Uppsala, Sweden) according to the instructions of the manufacturer. Twenty microliters of the PCR product and 15 pmol of sequencing primer p3 or p4 were used in sequencing reactions. The dispensation order was TGAGATCTAACGTACGTACGTACGTACGTACGTACG TACGT for the p3 sequencing reaction and TAGATCATCGATCGATCG for the p4 sequencing reaction.

TABLE 2. Oligonucleotides used in this work

Name	Sequence (5′→3′)	Purpose
p1	GCTAAATCCGTTGGTAATATCATGG	*parC* amplification
p2	CCAGATATTTCCGATAAACGGGC	*parC* amplification
p3	GGTAATTTCCATCCTCA	*parC* pyrosequencing
p4	GGAAAAATCGTGAAATTT	*parC* pyrosequencing
p5	ATCGCGGCTTAAGCCCCTTA	*umuC* probe DNA
p6	CTTTTCGCGTAAGCTCATTACTTG	*umuC* probe DNA
p7	AGGCGAAAGAATTGGGCTAT	*dnaE* probe DNA
p8	GCTCTTTTGCTGGAACTTCG	*dnaE* probe DNA
p9	GGCTGCAGGGGAAAAATGTTCCAAATCAGAAG	*hdiR* probe DNA
p10	CCGTCGACCTCGACAGCATAGATTGCTCC	*hdiR* probe DNA
p11	CCTCTAGACAATGATTTATCACGAAAAATTATTC	*dinP* probe DNA
p12	CGGTCGACGTTCAACTAAATCTGTATAACG	*dinP* probe DNA
p13	AGTTTGATCCTGGCTCAGGA	16S rRNA probe DNA
p14	GGTGTTACAAACTCTCGTGGT	16S rRNA probe DNA
p15	CGTCTAGAGCTTCAATCCTTATCAAGC	*umuC* cloning
p16	TACTGCAGGTAAGACATTTTGCCGTTTAG	*umuC* cloning
p17	GCCTGCAGGAACGCAGCCAATTAATTG	*umuC* cloning
p18	AAGTCGACAATTGAGATTGGCCCATTG	*umuC* cloning
p19	GGAATTCAGATACCAAGTTGGTCGTTTTTC	*umuC* complementation
p20	AATCTAGATAAAAGGAGTCACTACTATGTTC	*umuC* complementation
p21	CTGGATCCTTCTCAGGAAAACAATTAAAAACGATTC	HdiR purification
p22	CAATGTCGACCCTTGGTTCACGTGAATAATC	HdiR purification
p23	TAAATGGATCCCTTCACAAAATGAACTAGT	*hdiR* gel mobility shift
p24	HEX-CTGAGAACATAGTAGTGACTCC	*hdiR* gel mobility shift
p25	HEX-TCTCTAATAGCTGATTAAGATGC	*hdiR* gel mobility shift
p26	CTAGTTTAGTAAAGTTATAACTTTACTAAA	*hdiR* gel mobility shift
p27	AATTTTTAGTAAAGTTATAACTTTACTAAA	*hdiR* gel mobility shift
p28	CTAGAATATACTCATTTTTATCCATCTTTA	*hdiR* gel mobility shift
p29	AATTTAAAGATGGATAAAAATGAGTATATT	*hdiR* gel mobility shift

Database and bioinformatic tools used for identification of *S. uberis* genes and protein sequence comparisons. The sequence data used were obtained from the *S. uberis* sequencing group at the Sanger Centre (ftp://ftp.sanger.ac.uk/pub/pathogens/su/). Protein and DNA sequences for putative *umuC*, *hdiR*, *dinP*, and *dnaE* were downloaded from the Sanger Centre after identification of homologs by on-site BLAST (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/s_uberis). The sequences for the UmuC-like protein encoded by Tn*5252* (accession no. AAC98439), putative DinP of *S. pyogenes* (accession no. AAM80202), *S. pyogenes* DnaE (accession no. P0C0F2) (10), and HdiR of *L. lactis* (accession no. CAD89881) (58) were used as query sequences.

Annotations of *S. uberis* proteins were retrieved from an FTP site at ftp://ftp.sanger.ac.uk/pub/pathogens/bf/. The search of UmuC orthologs in different genomes was performed using the BLAST program at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) and the Comprehensive Microbial Resource at The Institute for Genomic Research (TIGR) website (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). The gene annotation of *Streptococcus mitis* NCTC12661 was taken from the TIGR Comprehensive Microbial Resource (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl).

RNA extraction and Northern blotting. *S. uberis* overnight cultures were diluted 1:500 in THY broth and grown at 37°C to an OD_{600} of 0.2 for the UV stress assay and an OD_{600} of 0.4 for the ciprofloxacin stress assay. Samples were withdrawn at 0, 30, and 60 min after UV treatment with 50 J/m^2 or 0, 30, 60, and 180 min after the addition of 0.5 μg/ml (0.5× MIC) ciprofloxacin. Cells were disrupted with 250 mg of 0.1-mm-diameter glass beads (Sigma) and a Fastprep FP120 homogenizer (ThermoSavant). RNA extraction was performed using a QIAGEN RNeasy Mini kit. DNA probes specific for *umuC* (encoding SUB0898), *dnaE* (encoding SUB1002), *hdiR* (encoding SUB0899), and *dinP* (encoding SUB1567) were amplified from strain ATCC BAA-854 by PCR using primer pairs p5/p6, p7/p8, p9/p10, and p11/p12, respectively. For Northern analysis, separation and transfer of RNA were carried out using a Latitude precast gel (1.25% Seakem Gold gel; Cambrex, CA) and a Hybond-XL uncharged nylon membrane (Amersham Pharmacia Biotech, CA) following the instructions provided by the manufacturer. Probe DNAs were labeled with [α-^{33}P]ATP (>92.5 TBq mmol^{-1}) using a DNA Megaprime labeling kit (Amersham Biosciences) following the manufacturer's instructions. Northern hybridization was carried out as described elsewhere (68).

The membrane was scanned and transcripts were quantified using a Fujifilm FLA-5100 scanner and AIDA software version 4.03.031 (Raytest Isotopenmessgeraete Gmbh, Germany). Differences in the amounts of RNA were corrected by quantifying the 16S rRNA amounts in each lane (primers p13/p14 for the probe).

Strain construction. Molecular cloning techniques were performed essentially as described by Sambrook and Russell (55). Restriction enzymes, DNA polymerase, and T4 DNA ligase were obtained from Promega (Madison, WI), Finnzymes (Espoo, Finland), and Roche (Mannheim, Germany), respectively. To create an in-frame deletion of the *umuC* gene in *S. uberis* ATCC BAA-854, a replacement recombination technique was used. The UmuC encoding region in ATCC BAA-854 was identified with the *S. uberis* BLAST server (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/s_uberis) using the UmuC encoded by streptococcal conjugative transposon Tn*5252* (accession no. AAC98439) as the query sequence.

PCR products were amplified from DNA regions flanking *umuC* with primers p15/p16 and p17/p18, digested with XbaI/PstI and PstI/SalI, respectively, and ligated to a XbaI/SalI-digested thermosensitive pGhost8 plasmid (36). The resulting plasmid, pEH55, was transferred to *E. coli* JM109 (Promega, WI), according to the manufacturer's instructions. Plasmid pEH55 was used to transform *S. uberis* cells in 10% glycerol with electroporation at 600 Ω and 25 kV/cm using a Bio-Rad Genepulser (29). The transformed cells were allowed to recover in THY broth supplemented with 6% sucrose at 28°C for 2 h followed by plating onto TSYE agar supplemented with 1 μg/ml tetracycline. Plasmid integration and excision were carried out as described by Biswas and coworkers (5), with integration and excision temperatures of 37.5°C and 28°C, respectively. Mutants were screened using colony PCR with primer pair p15/p18. The mutant strain carrying an in-frame deletion of *umuC* was assigned as EH58.

Complementation of the *umuC* deletion in strain EH58 was performed by pGhost8-mediated replacement of the *hdiR*-Δ*umuC*-ORF3-ORF4 operon with an intact operon. To accomplish this, a DNA fragment containing a *hdiR-umuC*-ORF3-ORF4 operon without its putative promoter region was amplified with the p19 and p20 oligonucleotides and cloned into pGhost8 as an EcoRI-XbaI fragment. Transformation, plasmid integration, and excision were performed as described above. The complemented EH58 derivative was assigned as EH80.

UV survival and mitomycin C sensitivity assays. UV sensitivity testing of *S. uberis* cells was performed on agar plates using spotting protocol adapted from Frees et al. (21). Briefly, overnight cultures were diluted 1:500 in fresh, preheated THY broth and incubated at 37°C to a final OD_{600} of 0.1. At this point, dilutions were made in phosphate-buffered saline, and 10 μl of the different dilutions were spotted on TSYE agar plates. Plates were exposed to UV light with an irradiance of 0 J/m^2 and 50 J/m^2 as described above. Colony-forming abilities of the wt and mutant strains were assessed after 1 day of incubation at 37°C.

Mitomycin C sensitivity testing was performed by spotting 10 μl of overnight cultures and appropriate dilutions made in phosphate-buffered saline on TSYE agar and TSYE agar supplemented with 25 ng/ml mitomycin C (Sigma). Colony-forming abilities of the wt and mutant strains were assessed after 1 day of incubation at 37°C.

Overexpression and purification of HdiR and analysis of self-cleavage. The *hdiR* coding region was amplified using primer pair p21/p22, digested with BamHI and SalI, and cloned into respective sites in pQE30 (QIAGEN). His_6-HdiR was purified from *E. coli* XL1-Blue carrying pQE30-6His-*hdiR* using the HisTrap HP 1-ml column according to the standard procedure recommended by Amersham Biosciences. The purified His_6-HdiR was used for DNA gel mobility shift experiments.

Self-cleavage of the His_6-HdiR was studied in the pH range of 6.0 to 10.0 using a buffer system containing either 50 mM Bis-Tris, Tris-HCl, or glycine. Briefly, each reaction containing 1 μg (20 μl) of purified HdiR was incubated at room temperature for 18 h. Reaction products were separated in a 12% Bis-Tris NuPage gel (Invitrogen) followed by staining with Coomassie brilliant blue R-250.

EMSA. A DNA fragment (167 bp) covering −157 to +10 relative to the *hdiR* start codon from *S. uberis* was generated by PCR using primer pair p23/p24. As a control DNA, an internal 154-bp fragment of *hdiR* (from +4 to +157) was amplified by PCR using primers p21/p25. The primers p24 and p25 carried HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein) fluorescence label at their 5′ end. Electromobility shift assay (EMSA) reactions (15 μl) were assembled by mixing the PCR-amplified fragment (35 ng) and the His_6-HdiR (0 to 80 ng) in gel shift buffer [20 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mg/ml bovine serum albumin, and 2 μg poly(dI-dC)]. Gel shift reactions were incubated at 25°C for 15 min followed by electrophoresis on a 5% polyacrylamide gel with 0.5× TBE (0.0445 M Tris, 0.0455 M borate, 1.25 M EDTA) at room temperature. Following electrophoresis, gels were scanned with a Fuji FLA-5100 scanner (Fuji Photo Film Co., Ltd., Japan) using an excitation laser at 532 nm, an output voltage of 400 V, and an LPG emission filter. Images were analyzed using the Aida Image Analyzer software v. 4.03 (Raytest GmbH, Straubenhardt, Germany).

Oligonucleotide pairs p26/p27 and p28/p29 with overhangs creating EcoRI and XbaI compatible ends, respectively, were annealed, treated with T4 polynucleotide kinase (MBI Fermentas), and ligated with T4 ligase (Roche) to XbaI-EcoRI cut pBluescript-II SK+ to obtain pBluescript-IR and pBluescript-ctrl, respectively. The M13 rev and M13 uni primers were used to amplify 230-bp fragments from pBluescript-IR and pBluescript-ctrl for the gel mobility assay. Reactions (15 μl) were assembled by mixing the PCR-amplified fragment (35 ng) and His_6-HdiR (0 to 80 ng) in gel shift buffer (20 mM Tris-HCl, pH 8.0, 60 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1 mg/ml bovine serum albumin, and 0.5 μg sonicated salmon sperm DNA). Gel shift reactions were incubated at 25°C for 15 min, followed by electrophoresis on a 5% polyacrylamide gel. Gels were stained with ethidium bromide, scanned with a Fuji FLA-5100 scanner (Fuji Photo Film Co., Ltd., Japan) using an excitation laser at 532 nm, an output voltage of 400 V, and an LPG emission filter. Images were analyzed using the Aida Image Analyzer software v. 4.03 (Raytest GmbH, Straubenhardt, Germany).

RESULTS

Induction of antibiotic resistance in *S. uberis* by UV light. Mutations leading to rifampin or ciprofloxacin resistance were used as an assay to determine whether *S. uberis* possesses a classical SOS response, as defined by increased mutation in response to UV light exposure. Rifampin resistance results from mutations in the β subunit of RNA polymerase (*rpoB*) that allow the polymerase to overcome the block (13). Ciprofloxacin resistance has been characterized from mutations in two genes, the topoisomerase gene *parC* and the gyrase gene *gyrA* (27).

UV-induced mutagenesis was examined by measuring the

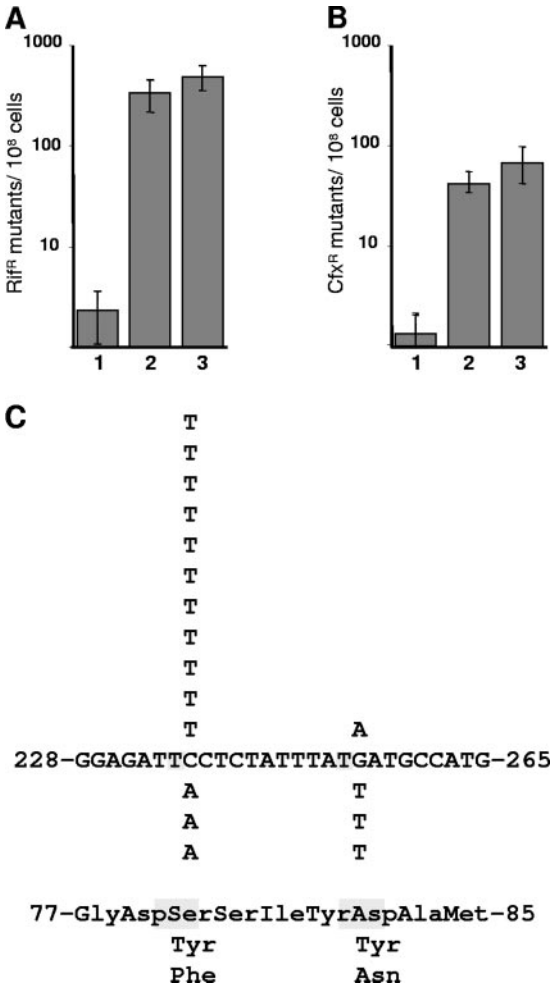

FIG. 1. Induction of mutagenesis to antibiotic resistance by UV light exposure in *S. uberis* ATCC BAA-854. (A) The proportions of Rif^r cells were determined in cultures that were either untreated (lane 1) or exposed to UV doses of 25 J/m^2 (lane 2) or 50 J/m^2 (lane 3). (B) The proportions of Cfx^r cells were determined in cultures that were either untreated (lane 1) or exposed to UV doses of 25 J/m^2 (lane 2) or 50 J/m^2 cells (lane 3). Results are presented as an average from at least three independent experiments, and error bars indicate standard deviations. (C) UV mutation spectra in the quinolone resistance-determining region of the *parC* gene. Sixty-four percent of mutants analyzed had a mutation in this region. Nucleotides 228 to 297 of the *parC* and the deduced amino acid sequence are shown in this figure. Mutations indicated above and below the nucleotide sequence correspond to transitions and transversions, respectively. The amino acid changes caused by these mutations are shown below the deduced amino acid sequence.

proportion of rifampin-resistant (Rif^r) or ciprofloxacin-resistant (Cfx^r) cells in the population. After UV induction at 25 J/m^2 and 50 J/m^2, the number of rifampin-resistant clones in overnight cultures increased 145- and 209-fold, respectively (Fig. 1A), and the number of ciprofloxacin-resistant clones in overnight cultures increased 31-fold and 48-fold, respectively (Fig. 1B). We determined the MIC of rifampin for nine individual UV-induced rifampin-resistant *S. uberis* clones by Etest. All rifampin-resistant clones were found to exhibit over 500-fold higher MICs than the wt strain (data not shown).

The nature of UV-induced mutations was analyzed in the *parC* gene by sequencing and pyrosequencing. First, we sequenced a fragment covering nucleotides +88 to +570 of the *parC* gene in 12 ciprofloxacin-resistant clones (data not shown). This area is part of the quinolone resistance-determining region, a 699-bp region of *parC* where ciprofloxacin resistance-promoting mutations have been characterized in *S. pneumoniae* and *S. pyogenes parC* (27, 46, 47). Based on sequencing results (data not shown) and previous work on other *Streptococcus* species (27, 46, 47), a 71-bp region (bases 228 to 299 of *parC*) that contains three frequently occurring mutations in streptococci was chosen as the template for pyrosequencing. DNA samples from 28 randomly selected individual ciprofloxacin-resistant clones were extracted and subjected to pyrosequencing. In 18/28 (64%) ciprofloxacin-resistant mutants, a mutation was observed in the 71-bp region responsible for previously described fluoroquinolone resistance-conferring amino acid substitutions in ParC (33). The mutation spectrum is shown in Fig. 1C. Most of the mutations observed were G:C→A:T transitions, constituting 67% of base changes observed. In most clones, the base substitution occurred in position 235 where 11 G:C→A:T transitions and 3 G:C→T:A transversions were detected (Fig. 1C). In one clone a transition was observed in position 246, whereas in three clones a transversion was observed in this position (Fig. 1C). In *E. coli*, G:C→A:T transitions represent the hallmark of UV mutagenesis (23).

From these results we conclude that *S. uberis* is capable of SOS mutagenesis.

Expression of SUB0898 and *hdiR* are induced in response to DNA damage. To further investigate the mutagenic response and the role of error-prone polymerases in *S. uberis*, we mined the genome sequence of *S. uberis* ATCC BAA-854, available at http://www.sanger.ac.uk/Projects/S_uberis/, for the presence of *umuC*, *dinP*, and *dnaE* genes. In addition, we searched for the presence of a gene encoding HdiR, a LexA-like regulator identified previously in *Lactococcus lactis* (58). BLAST searches revealed undefined gene products annotated as SUB0898, SUB1567, SUB1002, and SUB0899 with amino acid sequences 59%, 77%, 66%, and 33% identical to the UmuC-like protein of Tn*5252*, the putative DinP of *S. pyogenes*, the DnaE of *S. pyogenes*, and the HdiR of *L. lactis*, respectively. Northern analyses were performed using probes specific for genes encoding SUB0898 (putative UmuC, PolV subunit), SUB1567 (putative DinP, PolIV), SUB1002 (DnaE), and SUB0899 (HdiR) and RNA samples isolated from *S. uberis* cultures treated either with UV or with ciprofloxacin, which induces the SOS response in *E. coli* and *S. aureus* (14, 16, 67). Northern analyses of *S. uberis* indicated constitutive expression of putative *dinP* and *dnaE* genes under the conditions used (data not shown), whereas the expression of both putative *umuC* and *hdiR* was induced three- to sixfold following both UV and ciprofloxacin treatments (Fig. 2). Furthermore, *umuC*- and *hdiR*-specific probes detected two transcripts of the same sizes, approximately 1,100 bp and 3,000 bp, respectively, and exhibiting the same expression pattern (Fig. 2). Thus, the adjacent SUB0898 and SUB0899 genes encoding putative the UmuC-like protein and HdiR, respectively, were both induced under the DNA-damaging conditions used, indicating that the genes could be transcribed in the same transcript in *S. uberis*.

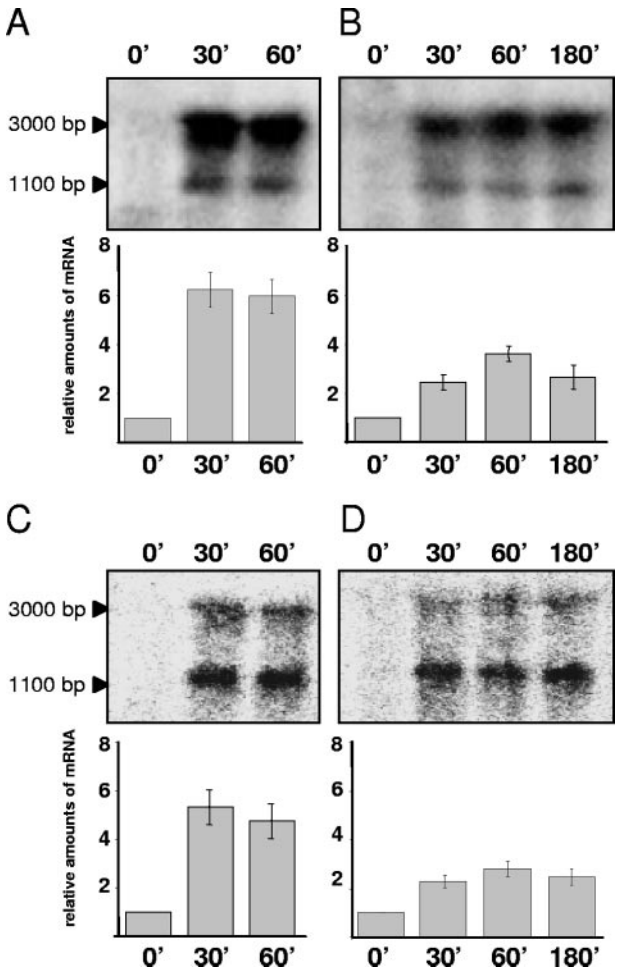

FIG. 2. Northern blot analysis of SUB0898 (*umuC*) and *hdiR* expression following exposure to DNA-damaging conditions. (A) *umuC* expression in ATCC BAA-854 before (0′) and 30 and 60 min after UV exposure of 50 J/m^2. (B) *umuC* expression in ATCC BAA-854 before (0′) and 30, 60, and 180 min after the addition of ciprofloxacin at 0.5 μg/ml. (C) *hdiR* expression in ATCC BAA-854 before (0′) and 30 and 60 min after UV exposure of 50 J/m^2. (D) *hdiR* expression in ATCC BAA-854 before (0′) and 30, 60, and 180 min after the addition of ciprofloxacin at 0.5 μg/ml. Bar diagrams show the relative mRNA induction ratios calculated by dividing the signal from the RNA sample by the signal from the RNA sample at time zero. Only the longer transcript detected with the *umuC* probe was quantified. RNA amounts were corrected after rRNA hybridization (data not shown), and results represent the mean values of two independent experiments with standard errors.

UV-induced mutagenesis requires SUB0898, a potential functional homolog of UmuC. To investigate the function of SUB0898 in *S. uberis*, we first retrieved the DNA sequence encoding SUB0898 and its flanking regions from http://www.sanger.ac.uk/Projects/S_uberis/ for sequence analyses. The 1,425-bp gene encoding SUB0898 is preceded by a 690-bp gene encoding the HdiR homolog SUB0899. The stop codon of *hdiR* is separated by 8 nucleotides from the start of the gene encoding SUB0898 (Fig. 3). The 384-bp open reading frame (ORF3) of SUB0897 overlaps with SUB0898 by 8 nucleotides and is followed by the 330-bp open reading frame (ORF4) of SUB0896 overlapping with SUB0897 by 8 nucleotides (Fig. 3). The *hdiR* gene is preceded by a well-conserved putative promoter region 38 nucleotides upstream and an inverted repeat (IR) structure representing a putative transcription terminator overlaps the stop codon of ORF4. Another IR is located between the putative promoter and start codon of *hdiR* (Fig. 3). This 26-bp IR is 96% identical to the structure located 12 bp upstream of the gene for a putative repressor that is 71% identical to the HdiR protein of *S. uberis* and has been annotated as spyM18_1150, SPs1040, M28_Spy0886, Spy1198, SpyM3_0840, and M6_Spy0903 in *S. pyogenes* strains MGAS8232, MGAS6180, SSI-1, M1 SF370, MGAS315, and MGAS10394, respectively. The organization of genes encoding SUB0899, SUB0898, SUB0897, and SUB0896 (Fig. 3) suggests that they could form an operon. SUB0897 and SUB0896 are homologous to uncharacterized proteins of *Streptococcus* species, whereas SUB0898 shares a high degree of homology to putative UmuC-like proteins from *Streptococcus* and related species with amino acid identities ranging from 48 to 64% (data not shown). The most homologous results, using SUB0898 as a query sequence, were found against putative ImpP/MucB/SamB family proteins from *Streptococcus agalactiae* 2603V/R (accession no. AE014250_6) and *S. pyogenes* MGAS10394 (accession no. AAT87297.1). For studying the function of SUB0898 in *S. uberis*, a mutant strain with an 1,350-bp in-frame deletion in the corresponding gene was constructed and named EH58. Compared to the wt, EH58 was sensitive to both UV light and the DNA-damaging agent mitomycin C (Fig. 4). In the absence of stress, EH58 exhibited a growth rate and final cell density similar to those of the wt strain (data not shown). UV-induced mutagenesis was examined by rifampin and ciprofloxacin resistance assays. Figure 5A and B show that UV light-induced mutagenesis in EH58 is dramatically decreased in both assays. The SUB0898 shows low amino acid identity (22%, in a 343-amino-acid overlap) to *E. coli* UmuC (accession no. NP_415702) and is almost equally homologous (22% identity in a 322-amino-acid overlap) with DinB (accession no. NP_414766). However, the UV-induced expression of the SUB0898 encoding gene, the UV- and mitomycin C-sensitive phenotype of EH58, and the almost complete lack of UV-induced mutagenesis in EH58 demonstrate that SUB0898 is a key element in mutagenic repair of UV lesions, and it suggests that SUB0898 is a functional homolog of UmuC in *S. uberis*. Thus, we named the gene encoding SUB0898 *umuC*. Despite several attempts, we have been unable to clone the *umuC* gene or the *umuC* operon along with its putative promoter region in a plasmid vector. Therefore, we cloned the entire *umuC* operon without a promoter into the pGh8-vector integration vector and used this construct to replace the *umuC* operon of EH58 with the wt operon in order to obtain EH80. Both UV and mitomycin C sensitivities as well as UV-induced mutagenesis in EH80 were complemented to the wt level (data not shown). This confirmed that the phenotype of EH58 was not due to secondary mutations unrelated to the *umuC* operon and that the *umuC* operon accounts for most of the mutagenic DNA repair in *S. uberis* cells exposed to UV light.

***umuC* and *hdiR* are part of a four-gene operon in *S. uberis*.** Northern analysis with a *umuC*-specific probe revealed a 3,000-bp transcript (Fig. 2) that is considerably longer than the 1,425-bp coding region predicted for *umuC*. Furthermore, a

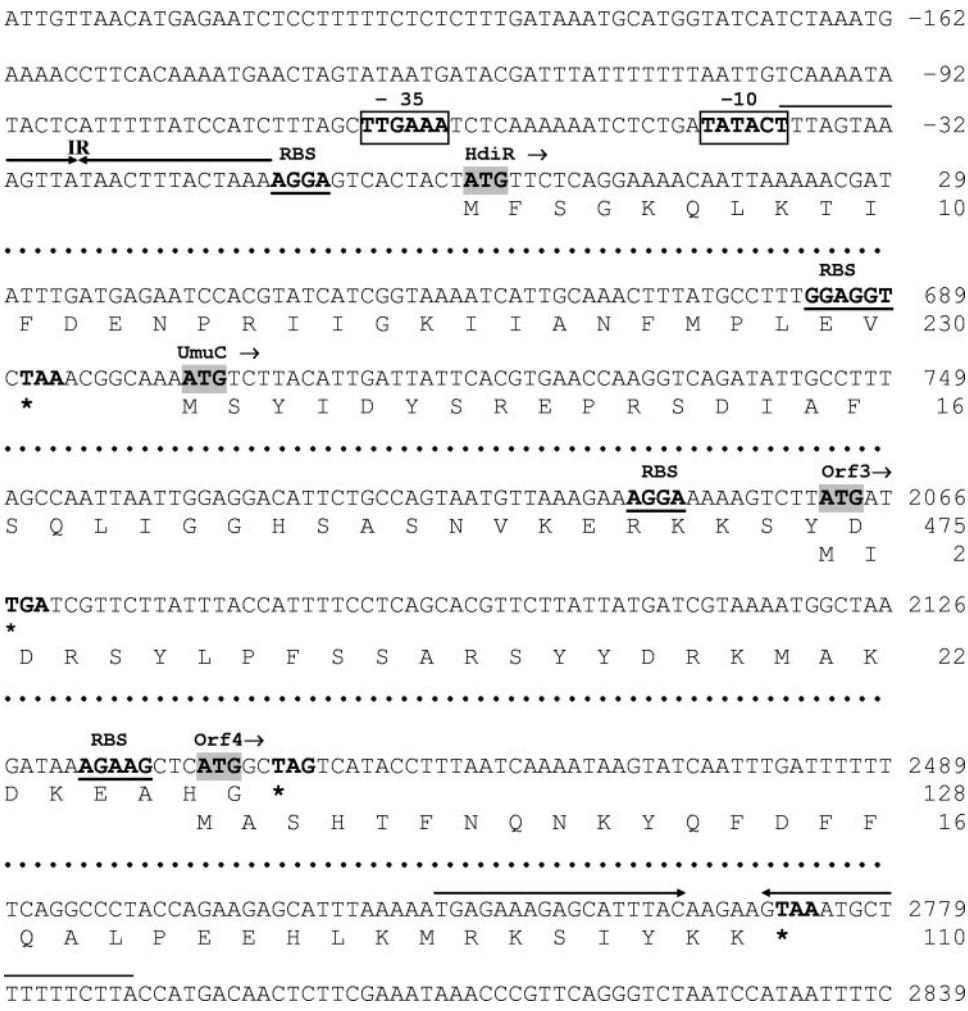

FIG. 3. Partial nucleotide and deduced amino acid sequences of the ATCC BAA-854 *hdiR-umuC* operon. The predicted −10/−35 hexanucleotides are boxed. The start codons are in boldface with dark background, and the putative ribosome-binding sites (RBS) are in boldface and underlined. Translation stop codons are marked with asterisks. The dotted areas represent gaps in the presented sequence. The 26-bp IR structure in the putative promoter region and the putative transcription terminator are marked with arrows.

3,000-bp transcript was detected using a probe specific for the adjacent gene encoding HdiR. Both probes also detected a shorter transcript of approximately 1,100 bp, but the relative signals of 3,000-bp and 1,100-bp transcripts were different with *umuC* and *hdiR* probes (Fig. 2). To confirm that *hdiR* and *umuC* are expressed in the same transcript, we analyzed RNA samples isolated from EH58 carrying a 1.35-kb deletion in *umuC* by Northern blotting with a *hdiR*-specific probe. Results shown in Fig. 6 demonstrate the presence of a single UV-induced *hdiR*-specific transcript in EH58, and the size of this transcript corresponds to the deletion in the *umuC* gene. Furthermore, the absence of the 1,100-bp transcript in EH58 suggests that the *hdiR-umuC* mRNA is processed from a site located in the deleted region of *umuC* in the wt. This would also explain the relatively weak signal of the 1,100-bp transcript with the *umuC*-specific probe compared to the *hdiR* probe (Fig. 2), resulting from only partial overlap of the *umuC* probe with this transcript. The predicted transcript size of *hdiR-umuC*-ORF3-ORF4 is approximately 2.8 kb. This corresponds with the size of the longer transcript detected with *hdiR* and *umuC* probes as a result of Northern analyses.

HdiR undergoes LexA-like self-cleavage in vitro and binds specifically to an IR located upstream of the *hdiR-umuC*-ORF3-ORF4 operon. According to sequence analysis, the *S. uberis* HdiR is 33% identical to its counterpart in *L. lactis* (58). In *L. lactis*, HdiR is a negative transcriptional regulator that has been shown to repress gene expression by binding to an IR structure located in the promoter regions of target genes (58). The *L. lactis* HdiR carries an N-terminal HTH motif that is essential for its function as a repressor, whereas the C-terminal half of the protein is not crucial for sequence-specific DNA binding. Analogous to LexA, the *L. lactis* HdiR requires RecA for self-cleavage in vivo, while self-cleavage occurs spontaneously in vitro at high pH (58). The capability of *L. lactis* for SOS-induced mutagenesis remains to be elucidated as does the biological role of HdiR in this organism. *S. uberis* HdiR carries a putative N-terminal HTH motif similar to that of *L. lactis* HdiR. In addition, amino acids Ala84, Gly85, Ser119, and

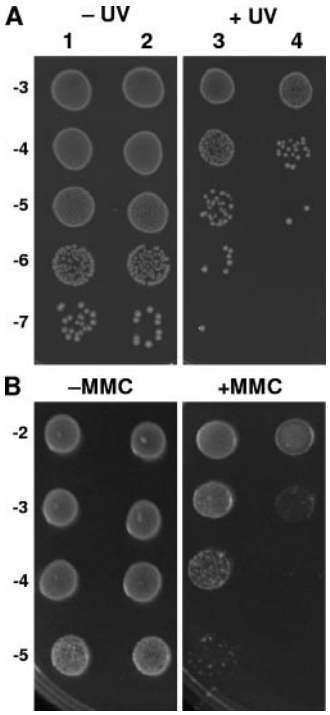

FIG. 4. Effect of absence of UmuC-like protein on DNA damage tolerance of *S. uberis*. (A) Survival of *S. uberis* ATCC BAA-854 and EH58 after UV exposure. ATCC BAA-854 (1 and 3) and EH58 (2 and 4) cultures were grown exponentially in THY and at an OD_{600} of 0.1, cultures were diluted, and 10 μl of each dilution was spotted on two TSYE agar plates. Plates either were not exposed (−UV) or were exposed to UV light of 50 J/m^2 (+UV) prior to incubation overnight at 37°C. The results presented are representative of several independent experiments. (B) Sensitivity of *S. uberis* ATCC BAA-854 and EH58 to mitomycin C (MMC). ATCC BAA-854 (1 and 3) and EH58 (2 and 4) cultures were grown overnight in THY, and 10 μl of overnight cultures and appropriate dilutions were spotted on TSYE agar plates without and with 25 ng/ml of MMC. Plates were grown overnight at 37°C. The results presented are representative of several independent experiments.

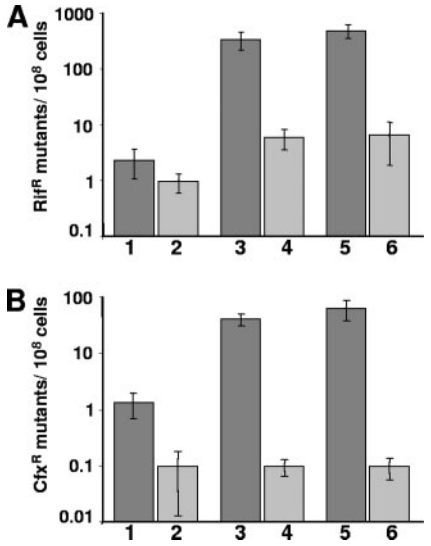

FIG. 5. Effects of absence of UmuC-like protein on UV-targeted mutagenesis. The proportions of (A) rifampin- and (B) ciprofloxacin-resistant cells in ATCC BAA-854 (dark gray bars) and EH58 (light gray bars) were determined in cultures that were either untreated (1 and 2) or were exposed to UV doses of (3 and 4) 25 J/m^2 and (5 and 6) 50 J/m^2.

Lys156, involved in the RecA-dependent self-cleavage of *E. coli* LexA (34, 59) and *L. lactis* HdiR (58), are also conserved in the HdiR of *S. uberis* (Ala117, Gly118, Ser150, and Lys187) (data not shown). We have not been able to inactivate the *recA* gene in *S. uberis* or to replace the wt *hdiR* gene with gene encoding uncleavable G118D HdiR (data not shown), which has prevented studies of HdiR self-cleavage in vivo. To study the function of *S. uberis* HdiR in vitro, we overproduced and purified HdiR as a His-tagged protein. The autodigestion at alkaline pH is a characteristic feature of LexA-like proteins undergoing RecA-mediated cleavage in vivo and has been documented for every LexA-like protein tested (23). For investigation of LexA-like self-cleavage, we incubated purified HdiR protein at various pHs and observed that self-cleavage of HdiR proceeded spontaneously at high pH, resulting in two products of ~14.6 and ~13.1 kDa (Fig. 7A). Next, we used EMSA to study the binding of HdiR to the upstream region of the *hdiR-umuC*-ORF3-ORF4 operon. To this end, the gel shift reactions containing purified HdiR and the DNA fragment spanning the region from −157 to +10 (promoter probe) or +4 to +157 (control probe) were assembled in binding buffer and the DNA complexes were separated in TBE gel, as described above. When scanning with an excitation wavelength specific for HEX-labeled DNA complexes, we observed that HdiR binds in a concentration-dependent manner to the promoter probe but not to the control probe (Fig. 7B). The additional band observed by EMSA is a single-stranded PCR product as demonstrated by denaturating the probe with formamide prior to electrophoresis (data not shown).

Next, we asked whether the 26-bp IR sequence inside the promoter probe is the target of HdiR binding. To examine this, the IR was cloned into pBluescript-II SK+ and used as a

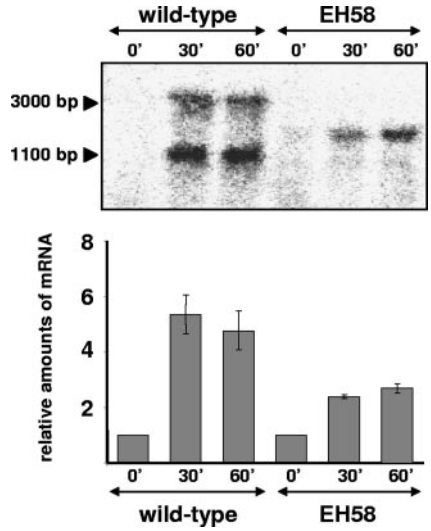

FIG. 6. Northern analysis of *hdiR* transcription in *umuC* deletion strain EH58. The *hdiR* expression in ATCC BAA-854 and EH58 before (0′) and 30 and 60 min after UV exposure of 50 J/m^2 is shown.

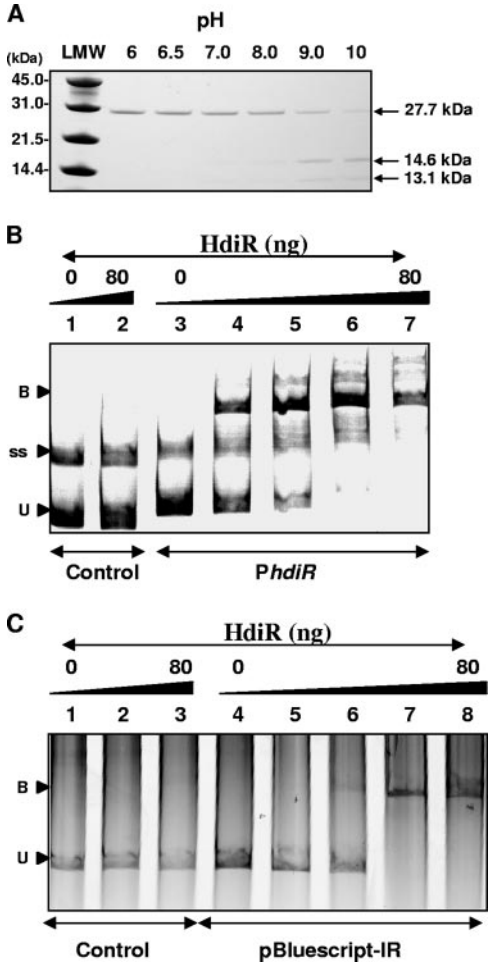

FIG. 7. In vitro analyses of HdiR self-cleavage and DNA-binding activity. (A) pH-dependent cleavage of the His_6-HdiR in the pH range of 6.0 to pH 10. Visualization of the autodigestion reactions containing 1,000 ng of the purified HdiR on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) gels stained with Coomassie brilliant blue. LMW refers to molecular weight standard (Bio-Rad). (B) Binding of the His_6-HdiR to the putative promoter region of *S. uberis hdiR-umuC*. Reaction mixtures contained 35 ng of PCR-derived DNA fragments from the *hdiR* internal region (negative control, lanes 1 to 2) or from the upstream region of the *hdir-umuC* operon (lanes 3 to 7) mixed with 0 ng (lanes 1 and 3), 20 ng (lane 4), 40 ng (lane 5), 60 ng (lane 6), or 80 ng (lane 2 and 7) of HdiR. Reactions were separated by 5% PAGE followed by scanning of the gel with a Fujifilm FLA-5100 scanner using an excitation laser at 532 nm and an LPG emission filter. Positions of unbound (or free) DNA (U), protein-DNA complexes (B), and the single-stranded DNA probe (ss) are indicated. (C) Binding of the His_6-HdiR to the pBluescript-II SK+ multiple cloning site containing a 26-bp control sequence without IR (lanes 1 to 3) or a 26-bp IR sequence (lanes 4 to 8) from the promoter region of *hdiR*. Reaction mixtures contained 35 ng of PCR-derived DNA fragments from pBluescript control (lanes 1 to 3) and from pBluescript-IR (lanes 4 to 8) mixed with 0 ng (lanes 1 and 4), 10 ng (lane 5), 20 ng (lane 6), 40 ng (lane 2), 60 ng (lane 7), and 80 ng (lanes 3 and 8) of HdiR. Reactions were separated in a 5% PAGE followed by staining with ethidium bromide and scanning with a Fuji FLA-5100 scanner using an excitation laser at 532 nm and an LPG emission filter. The positions of unbound (or free) DNA (U) and protein-DNA complexes (B) are indicated.

template in the PCR for the EMSA probe. In addition, a 26-bp sequence, located −96 to −70 from the start of *hdiR*, was cloned into pBluescript-II SK+ and used as a template for the control probe. EMSA using the probe amplified from pBluescript-IR and pBluescript-ctrl revealed that only the IR-containing DNA was retarded by HdiR (Fig. 7C).

Further EMSAs revealed that, parallel to *L. lactis* HdiR, the *S. uberis* HdiR retains its DNA-binding activity after self-cleavage (data not shown). We have made several attempts to inactivate *hdiR* in *S. uberis* using constructs that would lead to deletion of the entire gene or, alternatively, to deletion of the HTH motif of the protein. However, these efforts were not successful (data not shown). Inactivation of HdiR in *S. uberis* would presumably result in constitutive expression of the mutagenic gene cassette. Thus, it is possible that, unlike in *L. lactis* (58), this regulator cannot be inactivated, which hampers functional studies. However, the specific binding of purified HdiR to the IR sequence located between the putative promoter region and the start of *hdiR* strongly suggests that HdiR functions as a repressor of the expression of *hdiR-umuC*-ORF3-ORF4 and thereby regulates SOS mutagenesis in *S. uberis* in vivo.

The *umuC* operon and HdiR-binding sequence are conserved in *Streptococcus* genomes. Homology searches revealed genes encoding UmuC homologs with identities between 49 to 64% in *S. agalactiae* strains 2603V/R (65), H36B (66), and 18RS21 (66), in *S. pyogenes* strain MGAS10394 (2), in *Streptococcus sanguinis* strain SK36 (70), in *S. thermophilus* strains CNRZ1066 (8), LMG18311 (8), and LMD-9 (38), in *S. mitis* NCTC12661 (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi), and in *L. lactis* strains IL1403 (7) and SK11 (38). In addition, *S. pyogenes* 2812A transposon Tn*1207.3* (56), *S. pneumoniae* transposon Tn*5252* (41), *S. thermophilus* genomic island CIME19258 (48), and *L. lactis* plasmids pMRC01 (18) and pNP40 (43) may encode proteins with 64%, 57%, 58%, 50%, and 48% amino acid identities to *S. uberis* UmuC, respectively. Furthermore, in *S. pyogenes* MGAS10394, *S. mitis* NCTC12661, and *S. sanguinis* strain SK36, the genetic organization of the *hdiR-umuC*-ORF3-ORF4 operon is conserved (Fig. 8). In *S. agalactiae* sequences, a 132-bp open reading frame for an unknown protein is located between *hdiR* and *umuC*, and in Tn*1207.3*, two ORFs of 225 bp and 135 bp separate *hdiR* and *umuC* (Fig. 8). In the *S. thermophilus* and *L. lactis* genomes, *hdiR* and *umuC* are not in the same locus (Fig. 8). In Tn*5252* the organization of the four-gene operon is also conserved (Fig. 8). However, as noted previously (58), the product of ORF14 of Tn*5252*, exhibiting 63% amino acid identity to *S. uberis* HdiR, lacks a detectable N-terminal HTH motif according to the the Dodd-Egan algorithm (17) characteristic for all other HdiR homologs (Fig. 8).

Searches against completed genome sequences at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi revealed that *S. pyogenes* strains M1 GAS, MGAS10270, MGAS10750, MGAS2096, MGAS315, MGAS5005, MGAS6180, MGAS8232, MGAS9429, and SSI-1, *S. agalactiae* strains 515, A909, and NEM316, *Streptococcus suis* strain 89/1591, *Streptococcus mutans* strain UA159, and *S. pneumoniae* strains R6 and TIGR4 do not contain any gene encoding a UmuC homolog.

Next, we searched the upstream regions of streptococcal *hdiR* and *umuC* orthologs for the presence of sequences re-

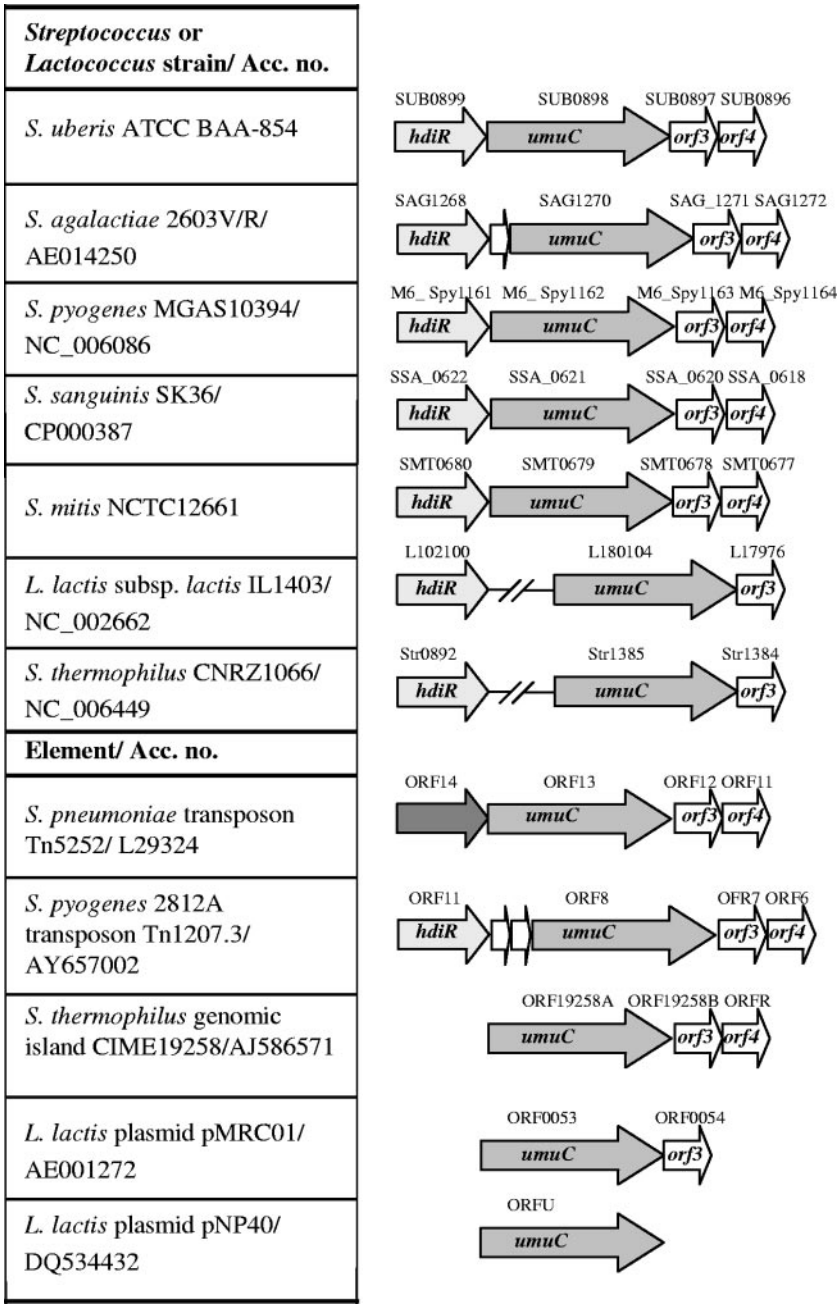

FIG. 8. Distribution of *umuC* in members of the *Streptococcaceae*. Schematic presentation of the *umuC* regions in *Streptococcus* and *Lactococcus* sequences. Dark gray indicates the ORF14 of Tn*5252* encoding a homolog of *S. uberis* HdiR lacking an N-terminal HTH motif.

sembling the binding site of *S. uberis* HdiR. DNA sequences showing homology (54 to 77% identity) to the 26-bp IR of *S. uberis* were found between putative promoter regions and start sites of several operons (Fig. 9).

Gene	Sequence	From start codon
SUB0899	TTTAGTAAAGTTATAACTTTACTAAA	(-12)
M6_Spy1161	TTTTGTTCAGTTATAACTAGACAAAA	(-11)
ORF11 (Tn1207.3)	TTTTGTTCAGTTATAACTAGACAAAA	(-11)
SMT0680	TTTTGTTACTTAATAAACTTACAAAA	(-10)
ORF14 (Tn5252)	TTTTGGTAGTTAATAAACTTACAAAA	(-9)
Str1385	TTTTGTTAGTTTATAAACTAACAAAA	(-11)
SAG1268	TTTTGTTAGTTTATAAAACAACAATA	(-16)
Str0892	TTATGTTAGTTTATTAACTTACGGAG	(-6)
SSA 0622	TGTGTAACTTTTAAAACGTTACATAG	(-9)

FIG. 9. Multiple-sequence alignment of DNA sequences identified upstream of *Streptococcus* genes resembling the binding site of *S. uberis* HdiR. A black background indicates nucleotides identical to the only known streptococcal HdiR-binding site, the IR structure preceding SUB0899.

DISCUSSION

Here, we show the existence of an SOS response-like DNA repair mechanism in a pathogenic member of the *Streptococcaceae* family. This activity includes mutation-promoting replication that requires the action of gram-positive-type UmuC

after induction with UV light. Furthermore, this study adds to previous work on HdiR (58), and we propose that this DNA-binding protein is a regulator of SOS mutagenesis as LexA is in other bacteria.

It was shown that UV light exposure induces mutations that confer antibiotic resistance in *S. uberis* ATCC BAA-854. Under DNA-damaging conditions, increased expression of the *hdiR-umuC*-ORF3-ORF4 operon was detected by Northern analyses, whereas no effect on *dinP* or *dnaE* expressions was observed. In addition to UV light, exposure to fluoroquinolone antibiotic ciprofloxacin also induced the expression of the *hdiR-umuC*-ORF3-ORF4 operon. A previous study revealed that the DNA polymerase encoded by *dnaE* in *S. pyogenes* is highly error prone, producing frameshift and point mutations in undamaged DNA as well as during TLS in vitro (11). In *M. tuberculosis* the SOS-induced DnaE2 was shown to be essential for evolution of antibiotic resistance in vivo (9). The functional study of DnaE proteins in gram-positive bacteria equipped with a single *dnaE* is hampered by the essentiality of the gene product (11). Our results demonstrate that the intact *umuC* gene is essential for UV-induced mutagenesis in *S. uberis* in vivo under the growth conditions used. The mutation types detected in the wt *S. uberis* strain after UV exposure were mainly transition-type base substitutions. This is in accordance with the transition mutations caused by PolV after UV induction in *E. coli* (30, 64). PolIV mainly produces −1 frameshift deletions in *E. coli* (69).

It was shown that the *umuC* gene is cotranscribed with a gene encoding the HdiR homolog as well as two unknown genes in *S. uberis*. Northern analyses suggest that the mRNA containing the *hdiR-umuC*-ORF3-ORF4 operon is processed within the *umuC* gene. The processing of the *hdiR-umuC*-ORF3-ORF4 transcript and possible differential stability of processing products could be a tool used by *S. uberis* to ensure that the amount of HdiR regulator exceeds other gene products of the operon and, possibly, results in more-efficient repression of the mutagenic operon. The novel regulator HdiR was previously characterized from *L. lactis* and was shown to function as a repressor that binds to an IR sequence located in the promoter regions of target genes (58). One target gene of HdiR was a *umuC*-like gene, present in *L. lactis* IL1403 but absent in another *L. lactis* strain, MG1363 (58). O'Driscoll and coworkers (44) recently reported the presence of a *umuC* ortholog in the *Lactococcus* plasmid pNP40 preceded by a repeat structure highly similar to the HdiR-binding sequence. EMSA revealed that the target sequence of *S. uberis* HdiR binding is located within an IR sequence that differs from the target of *L. lactis* HdiR. This is not surprising, since the two DNA-binding proteins are only 33% identical and the putative DNA-binding domains are not particularly well conserved (data not shown). DNA elements resembling the binding site of *S. uberis* HdiR were located upstream of *hdiR* and *umuC* genes in several *Streptococcus* species. However, it remains to be experimentally determined whether these DNA sequences represent functional operator sites of HdiR proteins in these organisms and whether the HdiR-binding site is conserved among streptococci. Database searches revealed that many, but not all, *Streptococcus* genomes encode proteins highly homologous to *S. uberis* UmuC. On the other hand, with the exception of *S. pneumoniae* genomes, all publicly available genome sequences from *Streptococcaceae* indicate the presence of an HdiR homolog (data not shown). However, the functions of all these HdiR-like proteins remain to be elucidated. Interestingly, while pathogenic and commensal members of the *Streptococcaceae* equipped with UmuC have conserved the organization of *hdiR* and *umuC* in a single operon, in the nonpathogenic dairy bacteria *L. lactis* and *S. thermophilus*, the operon organization has been split during evolution (Fig. 8.). It could be speculated that the presence of positive pressure in *L. lactis* and *S. thermophilus*, adapted to dairy environments, resulted in a separate regulator that gives the ability, e.g., for improved repression of mutagenic genes. *umuDC* homologs are often associated with mobile genetic elements of bacteria (49). In *Streptococcus* and *Lactococcus*, database searches revealed the presence of *umuC* homologs in plasmids, transposons, and genomic islands. Munoz-Najar and Vijayakumar (41) previously reported the presence of genes encoding UmuDC-like proteins in the pneumococcal conjugative transposon Tn*5252*. It was shown that plasmids carrying fragments of Tn*5252* restored UV-inducible mutagenic repair in *S. pneumoniae* (41). The UmuD-like protein encoded by Tn*5252* is highly homologous to HdiR; however, the lack of a HTH motif in the UmuD-like protein indicates functional diversification from HdiR. Small DNA damage-inducible and self-regulated gene cassettes capable of mediating adaptive mutagenesis have previously been identified from other bacteria including *Proteobacteria* and *Actinobacteria* (1, 20). However, to our knowledge the *hdiR-umuC*-ORF3-ORF4 operon enabling UV-induced adaptive mutagenesis in *S. uberis*, and potentially in other streptococci, represents the first SOS mutagenesis system that is not regulated by LexA.

In *E. coli* the function and activity of PolV is regulated at the transcriptional and posttranscriptional level, and it needs several other proteins to be capable of TLS. Despite the fact that UmuC is the catalytically active subunit, PolV requires two truncated UmuD subunits in order to be active (23). In gram-positive organisms, the functional homolog of UmuD remains to be identified despite the identification of candidates co-occurring with gram-positive UmuC-like proteins, like YqjX with YqjW in *B. subtilis* (19, 49). One of our future goals is to study the function encoded by the two uncharacterized genes present in *hdiR-umuC*-ORF3-ORF4 and address the question of why these genes co-occur with *umuC* among streptococci.

ACKNOWLEDGMENT

This study received financial support from the Academy of Finland (decision no. 211165 and 209136).

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